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Utilizing Protein-Lean Coproducts from Corn Containing Recombinant Pharmaceutical Proteins for Ethanol Production

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Protein-lean fractions of corn (maize) containing recombinant (*r*) pharmaceutical proteins were evaluated as a potential feedstock to produce fuel ethanol. The levels of residual *r*-proteins in the coproduct, distillers dry grains with solubles (DDGS), were determined. Transgenic corn lines containing recombinant green fluorescence protein (*r*-GFP) and a recombinant subunit vaccine of *Escherichia coli* enterotoxin (*r*-LTB), primarily expressed in endosperm, and another two corn lines containing recombinant human collagen (*r*-CIα1) and *r*-GFP, primarily expressed in germ, were used as model systems. The kernels were either ground and used for fermentation or dry fractionated to recover germ-rich fractions prior to grinding for fermentation. The finished beers of whole ground kernels and *r*-protein-spent endosperm solids contained 127−139 and 138−155 g/L ethanol concentrations, respectively. The ethanol levels did not differ among transgenic and normal corn feedstocks, indicating the residual *r*-proteins did not negatively affect ethanol production. *r*-Protein extraction and germ removal also did not negatively affect fermentation of the remaining mass. Most *r*-proteins were inactivated during the mashing process used to prepare corn for fermentation. No functionally active *r*-GFP or *r*-LTB proteins were found after fermentation of the *r*-protein-spent solids; however, a small quantity of residual *r*-CIα1 was detected in DDGS, indicating that the safety of DDGS produced from transgenic grain for *r*-protein production needs to be evaluated for each event. Protease treatment during fermentation completely hydrolyzed the residual *r*-CIα1, and no residual *r*-proteins were detectable in DDGS.

KEYWORDS: Corn; transgenic corn; recombinant protein; biofuels; dry-milling; degerming; distillers grains; corn biorefinery; maize

INTRODUCTION

Corn (maize) is regarded by many to be an ideal host to produce recombinant (*r*) pharmaceutical proteins and industrial enzymes because of its capability to produce *r*-proteins in high yield at low cost and having established production practices that provide for easy scale-up and practical and economic advantages in handling and processing (*1*−*3*). Typical *r*-protein recovery strategies extract the ground whole corn to recover only 1% of the grain mass and dispose of the remaining 99% as waste. Recently, it has been recognized that *r*-protein recovery may be integrated into the existing corn processing plants to utilize protein-lean coproducts and produce fuel ethanol and industrial chemicals by fermentation (*4*). Developing such a biorefinery platform would enhance the economics of *r*-protein recovery and enable efficient utilization of all corn components.

Because *r*-protein expression is often targeted to either the germ or endosperm of corn kernels, one could take advantage of grain fractionation methods that dry-mill grain into germ-, endosperm-, and fiber-rich fractions (*7*−*9*) or wet-mill it into relatively pure germ, fiber, starch, protein, and soluble (steep liquor) fractions (*4*). A simplified dry-milling process has been used to fractionate corn into germ-, bran-, and endosperm-rich fractions and worked well for recovering fractions enriched in *r*-proteins (*9*−*14*). In kernels containing *r*-protein expressed in germ, about 70% of the protein, can be recovered in 20−25% of the mass (*9*−*11*). The starch-rich endosperm could be directly used as a fermentation feedstock to produce biofuel and/or industrial chemicals. In kernels containing *r*-protein expressed in endosperm, for example, *r*-dog gastric lipase that might be used to treat cystic fibrosis, our simplified dry-milling procedure recovered 89% of the total enzyme in 70% of the grain mass as an endosperm-rich fraction (*9*, *14*). After extraction of the *r*-proteins from the endosperm-rich fraction, the spent solids may be utilized as a fermentation feedstock. Grain fractionation enriches the *r*-proteins, reducing the mass to be extracted as well as producing coproducts to be utilized in biofuel production and nonfood industrial applications such as paper, adhesives, and textiles.

Integrating grain-based *r*-protein production with biofuel production could enhance the economic viability by maximizing the overall value of the grain mass. Fuel ethanol is being produced in large quantities from corn (10.6 billion gallons per year, as of January 2010), and further expansion in ethanol production capacity is expected in coming years (*5*). The new renewable fuels standard (RFS) mandated by the U.S. Energy Independence and

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Table 1. Protocol for Laboratory-Scale Fuel Ethanol Production Using Simulated Industrial Operations and Parameters

step	operation
feedstocks	ground whole corn, endosperm fraction, or spent solids (500 g)
mashing and gelatinization	mix well with 1 kg of water incubate with 1.0 mL of α-amylase at 82 °C for 50 min with stirring (starch thinning) autoclave mash at 107 °C for 30 min (starch gelatinization)
liquefaction	adjust pH to 5.8 using 10 M ammonium hydroxide liquefy gelatinized starch with 1.0 mL of α-amylase at 82 °C for 2.5 h
simultaneous saccharification and fermentation	cool liquefied mash to 30 °C adjust pH to 5.0 with 10 M sulfuric acid add 1.0 mL of liquid glucoamlyase, 1.0 g of dry yeast, 0.5 g of urea, 20 μL of ammonia hydroxide, and 2 mg of Lactrol incubate at 32 °C and 120 rpm shaking for 60 h separate liquid beer by centrifugation dry wet cake and thin stillage at 50 °C for 48 h to produce dry DDGS

Security Act of 2007 calls for producing 15 billion gallons of grain-derived ethanol by 2015 (*6*). Therefore, such integration could mutually benefit *r*-protein recovery and fuel ethanol production; however, care must be taken when utilizing the coproducts of transgenic grains in feed applications. Some residual *r*-proteins will undoubtedly enter fermentation, and the effects of the *r*-protein on fermentation and the fate of *r*-proteins are unknown. Furthermore, in a typical dry-grind ethanol plant, one-third of the grain mass processed into ethanol remains as an unfermentable (protein- and fiber-rich) coproduct, distillers grains. Typically, the distillers dry grain with solubles (DDGS) is utilized as feed for livestock, particularly beef cattle. The feed industry will not likely tolerate any DDGS containing biologically active residual *r*-proteins. The objectives of this study were to evaluate the use of *r*-protein-lean coproducts to produce fuel ethanol, determine the impact of residual *r*-protein on ethanol production, and determine the fate of residual *r*-proteins.

MATERIALS AND METHODS

Materials. Four transgenic corn lines expressing *r*-proteins in endosperm (*r*-GFP-zn27, *r*-LTB) and germ (*r*-GFP-Glb1, *r*-CIα1) were used. The *r*-proteins were expressed in corn inbred lines having B73 background. Therefore, B73 corn was used as a nontransgenic control corn line to compare with the transgenic corn lines. Normal dent corn was used as a control corn in dry-milling and fermentation experiments .

Recombinant full-length human collagen type-I-α-1 (*r*-CIa1) chain (MW of 98.03 kDa) was expressed with its telopeptides and C-terminus folding enhancing peptide by using a globulin promoter directing *r*-protein accumulation into the germ; green fluorescent protein *r*-GFP-zn27 (MW of 27 kDa) was targeted to express in endosperm by using the 27zn promoter. Green fluorescent protein *r*-GFP-Glb1(MW of 25 kDa) was targeted to express in germ by using a globulin-1 (GIb1) promoter; antigenic subunit B of *Escherichia coli* heat-labile enterotoxin (*r*-LTB) consisting of homopentameric subunits (MW of 11.6 kDa) was targeted to express in endosperm by using an endosperm-specific 27zn promoter. The details of these transgenic events, *r*-LTB (*15*), *r*-CIα1 (*16*), and *r*-GFP (*17*), and sources have been previously published. The grain samples were hand-cleaned and stored at 4 °C until used.

The enzymes R-amylase SPEZYME Xtra (13642 R-amylase units/g) and G-ZYME 480 Ethanol (401 glucoamylase units/g) were obtained from Genencor International (Cedar Rapids, IA). Lactrol (462 g of virginiamycin/lb), an antibiotic extract, was obtained from PhibroChem (Ridgefield Park, NJ). Ethanol Red, dry yeast *Saccharomyces cerevisiae*, was obtained from Fermentis, Lesaffre Yeast Corp. (Headland, AL).

Dry Fractionation. The corn was conditioned to 21% moisture content by spraying water onto the corn placed in a sealed bag and allowing the moistened corn to equilibrate for 2.5 h. The tempered corn was dry-milled by using a laboratory Beal-type drum degermer at 50% speed followed by our simplified dry-milling method as has been previously described (*9*, *10*).

Feedstock Preparation. Three types of feedstocks were prepared for fermentation: (i) traditional dry-ground whole kernels; (ii) dry-milled (degermed) endosperm-rich solids; and (iii) *r*-protein extracted spent-solids of endosperm. Whole grains and dry-milled endosperm fractions were ground into flour by using a laboratory grinder (Nutrimill, Salt Lake City, UT) operating in medium grinding mode.

r-Protein-extracted (spent) solids were prepared by using protein extraction protocols specific for each *r*-protein as described by Zhang et al. (*16*) for *r*-CIα1 and by Moeller et al. (*15*) for *r*-LTB proteins. For extracting *r*-CIα1, ground corn flour was extracted twice by mixing with extraction buffer (0.1 M phosphoric acid, 0.15 M sodium chloride, pH 1.8) at a 1:5 w/v ratio for 1 h at room temperature. For extracting *r*-LTB, the ground solids were extracted twice by mixing with extraction buffer (25 mM sodium phosphate, pH 6.6, containing 100 mM NaCl, 0.1% Triton X-100 (v/v), 1 mM ethylenediaminetetraacetic acid, 10 μg/mL of leupeptin, 0.1 mM serine protease inhibitor Perfabloc SC) for 2 h at 37 °C. *r*-GFP was extracted by using a method similar to the method used for *r*-LTB proteins. The extracts were separated from the insoluble material by centrifuging (5000*g*, 10 min, 25 °C), and the spent solids were used as fermentation feedstock.

Ethanol Production. The laboratory-scale fermentation procedure described in Wang et al. (*18*), which closely simulates industrial fermentation, was used (**Table 1**). A 500 g ground corn sample (listed in **Table 2**) was slurried with deionized water in a 2 L flask to obtain 30% solids. The slurry was mixed with a top-drive stirrer and liquefied by using 1 mL of α-amylase (SPEZYME Xtra, Genencor Int., Rochester, NY) at 82 °C for 50 min. The partially liquefied slurry was autoclaved at 107 °C for 50 min to gelatinize the starch. The gelatinized starch was further liquefied by incubation with 1 mL of α-amylase at 82 °C for 150 min in a water bath. The liquefied mash was cooled to 30 °C (pH as is). Simultaneous saccharification and fermentation (SSF) was carried out by adding 500 mg of urea, 2.0 mg of Lactrol, 1.0 mL of liquid glucoamlyase, and 1.0 g of dry yeast and incubating at 32 °C for 60 h with shaking at 120 rpm in an incubator−shaker. After fermentation, the yeast was inactivated to prevent ethanol loss by heating in a water bath at 70 °C for 20 min in tightly stoppered flasks.

For protease treatment, the same fermentation procedure was carried out with endosperm-rich solids while adding 0.05% Protex 6 L, a bacterial serine protease derived from *Bacillus licheniformis* (Genencor Int.), on corn weight basis during SSF.

Fermented Beer Composition. A 50 mL sample of the finished beer was centrifuged at 5000*g* for 15 min and analyzed for ethanol, acetic and lactic acids, and glycerol concentrations and residual sugar profiles by using a Waters high-pressure liquid chromatograph (Millipore Corp., Milford, MA) equipped with a Waters model 401 refractive index detector as described by Prachand et al. (*19*). A 20 μL sample was injected, and the compounds were separated by using a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) with 0.012 N sulfuric acid as mobile phase at 0.8 mL/min flow rate and 65 °C column temperature.

Chemical Analysis. The *r*-protein contents were estimated by using an enzyme-linked immunosorbent assay (ELISA) as described in Zhang et al. (*16*) for *r*-CIα1 and in Moeller et al. (*15*) for *r*-LTB proteins. The *r*-GFP contents were determined by measuring fluorescence activity of the

Table 2. Partitioning of *r*-Proteins in Germ-, Endosperm-, and Pericarp-Rich Fractions Separated by Dry-Milling[a]

		r-protein concentration (μg/g of tissue)			*r*-protein distribution (%)		
corn line	whole kernel *r*-protein content (μg/g of kernel)	germ	endosperm	bran	germ	endosperm	bran
r-CIα1 grain	3.7	9.2	1.8	1.4	62.6	35.0	2.4
r-GFP-Glb1 grain	12.7	18.9	3.6	1.8	59.9	37.8	2.3
r-GFP-Zn27 grain	26.5	8.7	28.2	9.4	7.7	88.0	4.4
r-LTB grain	26.6	13.6	32.0	10.4	12.2	84.3	4.5

[a] Based on summation of total *r*-protein contents of all three fractions.

Table 3. Mass Yields and Oil Recoveries of Fractions Separated by Simplified Dry-Milling[a]

	germ-rich fraction			endosperm-rich fraction		
corn line	germ yield (%)	oil content (%)	oil recovery[b] (%)	endosperm yield (%)	oil content (%)	oil recovery[b] (%)
r-CIα1 grain	24.1 a	11.9 a	58.5 b	68.6 a	2.0 a	27.8 a
r-GFP-Glb1 grain	21.6 a	12.6 a	70.2 a	69.2 a	1.1 a	20.1 b
r-GFP-Zn27 grain	19.3 a	14.1 a	73.0 a	67.9 a	1.1 a	20.5 b
r-LT-B grain	20.6 a	16.0 a	70.4 a	65.3 a	1.3 a	18.7 b
Dent Cuba grain	25.7 a	12.7 a	69.9 a	66.8 a	1.5 a	21.4 b
B73 grain	21.5 a	13.1 a	62.3 b	71.1 a	1.2 a	21.7 b
LSD	6.1	4.6	6.5	6.6	0.8	5.6
p value	0.2486	0.4064	0.0123	0.4471	0.2063	0.0465

[a] Means in the same column with the same letter are not significantly different ($p < 0.05$). [b] Oil recovery is the percentage of oil recovered in fractions based on total kernel oil.

samples by using a spectrofluorometer as described in Shepherd et al. (*10*, *17*). Corn samples and DDGS were further ground by using a household coffee grinder and sieved through a 50-mesh sieve (0.30 mm opening) prior to determining contents of *r*-protein and other chemical constituents. Western blotting and antibody detection for *r*-LTB, *r*-GFP, and *r*-CIα1 were carried out as described by Zhang et al. (*16*) for *r*-CIα1 and by Moeller et al. (*15*) for *r*-GFP and *r*-LTB proteins.

Crude protein contents were determined by using the Dumas nitrogen combustion method with an Elementar Vario MAX CN analyzer (Elementar Analysesysteme GmbH, Hanau, Germany). Starch contents were determined by using the amyloglucosidase/α-amylase enzymatic method 79-13 (*20*) with an enzyme kit (Megazyme, Wicklow, Ireland). Crude free fat contents were determined by using AACC method 30-25 (*20*) with the Goldfish apparatus (Labconco Corp., Kansas City, MO). Moisture contents were determined by using the 130 °C convection oven method 44-19 (*20*); whole kernel moisture contents were determined by using the 103 °C convection oven method 44-18 (*20*).

Statistical Analysis. All treatments and analyses were conducted in triplicate. Data were analyzed by using analysis of variance with JMP v. 6.0.2 statistical software (SAS Institute, Inc., 2006). Least significant differences (LSD) were determined by using the Tukey−Kramer HSD test at the 5% significance level.

RESULTS AND DISCUSSION

Recovering Protein-Rich Fractions. The partitioning of the *r*-proteins in dry-milled germ-, fiber-, and endosperm-rich fractions is shown in **Table 2**. In grains where *r*-protein expressions were targeted to the germ, our simplified dry-milling procedure recovered germ-rich fractions with 68 and 54% recoveries of the total *r*-CIα1and *r*-GFP, respectively, in 20−26% kernel mass. These germ recoveries from the transgenic grains compared favorably with those of a nontransgenic dent corn (Dent Cuba) and the inbred (B73) in which the *r*-protein events were expressed. Such recovery is beneficial because it enriched the proteins ∼2.5-fold in one-fourth of the original kernel mass, which reduces the cost of protein recovery because of the higher concentrations of the target species and reduced mass to process. The fractionation efficiencies were consistent with previous dry-milling studies of Zhang et al. (*11*), who recovered about 60% of the total *r*-CIα1 in 25% of the kernel mass.

In corn where the *r*-protein expression was targeted to endosperm, dry-milling recovered about 78 and 80% of the total *r*-LTB and *r*-GFP, respectively, in the endosperm-rich fractions. About 73% of the germ was removed in the endosperm-rich fraction by dry-milling (**Table 3**), which reduced the mass for protein extraction as well as amounts of nontarget proteins, from which the *r*-protein must be purified, and oil, which complicates purification. Corn germ is rich in water-soluble proteins (enzymes) and fat to support a germinating corn embryo. The endosperm-rich fraction contained only 1.1−2.0% oil, whereas the original grain contained 3.8−4.9% and the germ-rich fractions contained 11−16% oil. Crude protein contents varied slightly for germ (13.7−17.0%) and endosperm (7.3−9.6%) fractions. Corn germ proteins are more water-soluble and difficult to separate from the target *r*-protein, whereas the endosperm proteins are primarily alcohol-soluble storage prolamins and, therefore, were not readily extracted from the endosperm fraction when using the selected extraction buffer or aqueous extraction solutions. The starch contents also varied greatly among the fractions. Of the total starch in the kernel, 86−91% was recovered in the endosperm-rich fraction and 5−13% in germ-rich fraction (data not shown).

By using GFP as a tissue marker, Shepherd et al. (*10*) demonstrated that the endosperm-rich fraction from our simplified dry-milling procedure contained 4% germ tissue, but the germ-rich fraction contained 28% germ, 20% endosperm, and 52% non-endosperm and nonembryo tissue (bran). It is easier to get high-purity endosperm than high-purity germ and bran. The starch-rich endosperm fractions produced in the present study were then evaluated as feedstocks for producing fuel ethanol.

Utilizing Protein-Lean Fractions. Ground whole kernels of transgenic and nontransgenic grains were evaluated in dry-grind ethanol production, and the amounts of *r*-proteins were monitored after each processing step. Both *r*-GFP and *r*-LTB proteins were inactivated during the liquefaction steps of starch thinning and gelatinization; however, residual *r*-CIα1 protein was found in the mash that entered the fermentation step (data not shown).

After fermentation, the ethanol concentrations of the beers did not significantly differ among feedstocks produced from transgenic (127−139 g/L) and nontransgenic (129−137 g/L) corn lines

Table 4. Ethanol Yields and Chemical Compositions of Fermented Beers Produced from Ground Whole Kernels and Spent Endosperm Solids[a]

feedstock/corn line	ethanol (g/L)	glycerol (g/L)	lactic acid (g/L)	soluble sugars (g/L)	ethanol yield (%)	ethanol efficiency (%)
Ground Whole Kernels						
r-CIα1 grain	127.1	0.75	0.15	1.4	28.2	77.1
r-GFP-Glb1 grain	135.4	0.70	0.00	0.9	30.1	82.3
r-GFP-Zn27 grain	138.6	0.73	0.23	1.0	30.8	84.2
r-LTB grain	126.7	0.72	0.15	1.5	28.2	77.1
Dent Cuba grain	137.1	0.98	0.00	0.9	30.5	83.3
B73 grain	129.1	0.56	0.13	1.2	28.7	78.4
mean	132 ± 11.2	0.7 ± 0.1	0.1 ± 0.1	1.2 ± 0.3	29.4 ± 2.5	80.3 ± 3.2
***r*-Protein-Spent Endosperm Solids**						
r-CIα1 grain	147.6	0.68	0.00	1.8	32.8	75.9
r-GFP-Glb1 grain	155.4	0.72	0.00	1.2	34.5	79.8
r-LTB grain	138.1	0.85	0.00	1.1	30.7	71.0
r-GFP-Zn27 grain	151.3	0.79	0.16	1.7	33.6	77.7
Dent Cuba grain	150.2	0.72	0.00	1.4	33.4	77.2
B73 grain	153.6	0.81	0.25	0.9	34.1	78.9
mean	149 ± 9.1	0.8 ± 0.1	0.07 ± 0.1	1.4 ± 0.4	33.2 ± 2.1	72.5 ± 3.1

[a] Theoretical ethanol yield (g of ethanol/100 g of feedstock) = (dry wt of starch × mol wt of glucose/mol wt of starch) × (2 × mol wt of ethanol/mol wt of glucose). Theoretical ethanol yield for feedstock of ground whole grain was 36.6% based on 68.9% starch content and that of endosperm was 44.2% based on 80.6% starch content.

(**Table 4**). The slight variations observed among ethanol yields were likely due to normal variations in kernel composition. Overall, the *r*-proteins did not negatively affect ethanol production.

When the effects of grain fractionation on fermentation were compared, ethanol yield significantly differed between feedstocks that used ground whole grain or their dry-milled endosperm-rich fractions (**Table 4**). Ground whole grain fermentation produced 127−139 g/L ethanol, whereas the endosperm-rich solids produced 138−155 g/L ethanol. Ground whole grain produced 29.4% mean ethanol yield, whereas the endosperm-rich fraction produced a 33.2% mean ethanol yield when using the same amounts of starting materials. Removing the germ- and fiber-rich fractions enriched the starch content of the fermentation mash and thereby increased the ethanol concentration of the finished beer. The endosperm-rich solids contained higher initial starch contents (79−86%) compared with whole kernels (62−68%). If total conversion of the kernel starch to ethanol is considered, the endosperm-rich fraction produced less ethanol yield than did ground whole kernels. This was attributed to (i) loss of 10−13% of the total starch in the germ and pericarp fractions and (ii) lower ethanol production efficiency for the endosperm-rich solids (**Table 4**).

Experiments using endosperm-rich fractions as feedstocks and their *r*-protein-extracted spent solids indicated that the buffers used to extract *r*-protein did not negatively affect either enzyme or yeast function during hydrolysis and fermentation, respectively (data not shown). The two feedstocks (endosperm-rich fractions and their *r*-protein-extracted spent solids) produced fermented beers with similar ethanol yields and compositions. This indicated that *r*-protein-extracted solids can be directly used for fermentation without removal of residual buffer by washing, if the same buffers used in the present study are used for *r*-protein extraction.

The amounts of residual sugars, lactic acid, and glycerol in the fermented beers were similar to those produced from ground whole kernels, endosperm-rich fractions, and the *r*-protein-extracted spent solids (**Table 4**). Residual glucose was undetectable in the beer, indicating complete fermentation of free sugars. The concentrations of acetic acid (< 0.1% w/v) and lactic acid (< 0.25% w/v) were below critical limits, indicating no bacterial contamination occurred during fermentation. The concentrations of glycerol (< 0.98% w/v) were also below the threshold limit for yeast stress. Yeast produces glycerol when stressed, for example, in high sugar or ethanol concentrations that inhibit yeast growth. The chemical composition of the beer confirmed that (i) low sugar concentration was maintained during SSF, avoiding osmotic stress on yeast; (ii) degermed endosperm fractions provided adequate supply of required nutrients for proper yeast function; (iii) any residual buffers used to extract *r*-protein did not affect fermentation; and (iv) residual *r*-proteins did not adversely affect yeast or enzyme function during SSF.

Compositions of Distiller's Grains. Table 5 summarizes the amounts of *r*-proteins in various fractions after dry-milling, protein extraction, and ethanol fermentation. DDGS contained no biologically active *r*-GFP or *r*-LTB proteins after fermentation of the ground whole transgenic grains or their *r*-protein-extracted spent solids. As discussed previously, both *r*-GFP and *r*-LTB proteins were inactivated during the liquefaction step; thus, no biologically active *r*-GFP or *r*-LTB was found in the fermentation mash before or after fermentation. Residual *r*-CIα1, however, was found in DDGS, although *r*-CIα1 is heat-sensitive (*16*). Because *r*-GFP and *r*-LTB proteins are functional proteins, they were quantified for their biological functionalities by using fluorescence and ELISA methods, respectively. *r*-CIα1, on the other hand, is a structural protein and thus was quantified for physical presence. Even the denatured forms of *r*-GFP and *r*-LTB proteins were not detectable in DDGS by using Western blots (**Figure 1B**,**C**), indicating these proteins were completely denatured during ethanol production and thus the antigenic portion of the protein was no longer intact to bind with antibodies. Yeast cannot metabolize protein (*21*) and, therefore, does not explain the disappearance. We attributed the absence of these *r*-proteins in Western blots to one or more of the following: (i) degradation of the *r*-proteins due to the presence of protease contamination in the enzymes used for starch liquefaction and scarification; (ii) cross-linking or aggregation of these *r*-proteins as a result of high temperatures used in starch liquefaction, distillation and DDGS drying steps such that they can no longer be extracted for Western blotting.

Table 6 shows the residual oil, crude protein, and starch components of DDGS. Oil content was much lower in the DDGS

Table 5. Fates of Residual *r*-Proteins in DDGS Produced from Transgenic Ground Whole Kernels, Endosperm-Rich Solids, and *r*-Protein Spent Endosperm Solids

corn line/ feedstock fraction	initial *r*-protein in feedstock		residual *r*-protein in DDGS	
	r-protein concentration (μg/g of tissue)	*r*-protein distribution[a] (%)	*r*-protein concentration (μg/g of tissue)	*r*-protein distribution[b] (%)
		***r*-Clα1 gGrain**		
ground whole kernel	3.7	107.7	2.9	30.7
endosperm-rich solids	1.8	33.5	1.7	17.9
r-protein-extracted spent endosperm solids	0.1	3.6	0.1	0.8
		***r*-GFP-Glb1 Grain**		
ground whole kernel	12.3	124.1	nd[c]	nd
endosperm-rich solids	3.6	41.7	nd	nd
r-protein-extracted spent endosperm solids	0.9	2.9	nd	nd
		***r*-GFP-Zn27 Grain**		
ground whole kernel	26.5	145.3	nd	nd
endosperm-rich solids	28.0	89.5	nd	nd
r-protein-extracted spent endosperm solids	2.7	8.6	nd	nd
		***r*-LTB Grain**		
ground whole kernel	26.5	96.2	nd	nd
endosperm-rich solids	32.0	84.3	nd	nd
r-protein-extracted spent endosperm solids	5.3	12.0	nd	nd

[a] Initial *r*-protein distribution was calculated on the basis of **Table 2**. [b] Residual *r*-protein was calculated relative to initial *r*-protein content of feedstock. [c] Not detected.

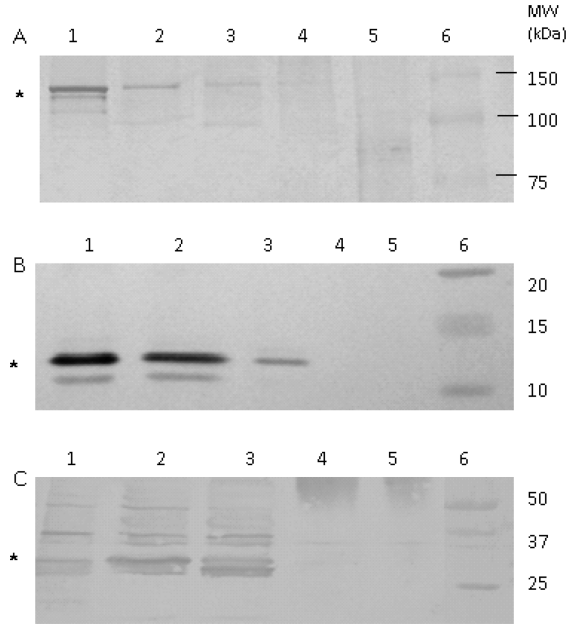

Figure 1. Western blots of corn samples and their DDGS containing *r*-Clα1 (**A**), *r*-LTB (**B**), and *r*-GFP-Zn27 (**C**) proteins. Sample lanes: ground whole kernels (lane 1); dry-milled endosperm (lane 2); *r*-protein-extracted spent endosperm solids (lane 3); DDGS of dry-milled endosperm without protease treatment (lane 4); DDGS of dry-milled endosperm with protease treatment (lane 5); molecular weight marker (lane 6). The *r*-proteins are indicated by asterisks on the left side of each picture (*r*-Clα1 = ~120 kDa; *r*-LTB monomer = 11.7 kDa; *r*-GFP-Zn27 = 27 kDa).

produced from dry-milled endosperm-rich feedstock (3.3−7.0%) than in that produced from traditional whole ground corn (9.1−13%). This is advantageous because the high fat content of DDGS without front-end fractionation contributes little feed value and is detrimental to pork quality. A similar trend was observed for protein contents of the DDGS, although the differences were not as large as for oil content. DDGS of fractionated kernels (endosperm) contained a higher amount of residual starch (6.3%) than DDGS prepared from ground whole kernels (< 1%). The high amount of unfermented residual starch was likely due to higher starch contents (80.6%) of endosperm-rich fractions than of ground whole kernels (68.9%). The conditions used for liquefaction were optimum for whole-grain distilleries and may not have been optimal for feedstocks containing higher starch contents such as *r*-protein-extracted endosperm solids.

Adding protease (Protex 6 L) during SSF hydrolyzed the *r*-proteins including *r*-CIα1; no residual *r*-protein was found in DDGS (**Figure 1**; **Table 7**). Protease is frequently used in dry-grind ethanol production to increase fermentation rate and production of ethanol by breaking down matrix protein surrounding starch granules, which prevents access of starch-saccharifying enzymes. Protease treatment would ensure that intact *r*-proteins are not present in DDGS produced from the transgenic grains. Furthermore, protease treatment increased the mean ethanol concentration in the fermented beer from 149 to 156 g/L and the mean ethanol yield from 33.2 to 34.6% when dry-milled endosperm-rich solids were used as feedstock (**Table 7**).

Effects of Fractionation on Fermentation. Murthy et al. (*22*) indicated that germ separation prior to fermentation reduced ethanol yield by 1.5% and attributed the reduction to limited availability of fatty acids required for yeast growth. The present study, however, indicated that higher starch concentration probably accounts for the lower efficiency of converting starch to ethanol. Our speculation is supported by the following. First, the glycerol concentration of the finished beer was below critical limits, indicating no nutritional stress on yeast. In general, limited fatty acids nutritionally stress yeast and elevate glycerol concentration of fermented beer (*21*, *22*). Our dry-milled endosperm-rich fractions contained 1.1−1.8% oil, which should have been sufficient to provide the necessary fatty acids needed for normal yeast metabolism. Second, the active dry state of distillery yeast can provide a higher concentration of yeast that may not require

Table 6. Compositions and Yields of DDGS Produced from Dry-Ground Whole Kernels and Spent Solids of Endosperm-Rich Solids[a]

feedstock/ corn line	recombinant protein (μg/g)	crude free fat (%)	protein (%)	starch (%)	DDGS yield (%)
Ground Whole Kernels					
r-CIα1 grain	2.9	13.0 a	40.9 a	1.3 d	34.4 a
r-GFP-Glb1 grain	0.0	11.9 a	37.2 ab	1.3 d	29.2 a
r-LTB grain	0.0	12.9 a	37.2 ab	2.3 c	31.1 a
r-GFP-Zn27 grain	0.0	12.2 a	37.1 ab	1.2 d	34.4 a
Dent Cuba grain		10.7 a	42.5 a	0.9 d	31.5 a
B73 grain		11.6 a	38.4 ab	1.1 d	33.1 a
mean		12.0 ± 1.8	38.9 ± 1.8	1.4 ± 0.5	32.3 ± 2.7
***r*-Protein-Spent Endosperm Solids**					
r-CIα1 grain	0.1	6.2 b	38.0 ab	3.9 b	28.6 b
r-GFP-Glb1 grain	0.0	5.0 b	38.4 ab	4.2 b	24.3 c
r-LTB grain	0.0	2.8 b	32.3 b	5.6 a	33.1 a
r-GFP-Zn27 grain	0.0	3.9 b	33.5 b	3.8 b	26.8 bc
Dent Cuba grain		5.6 b	37.2 ab	3.3 bc	27.1 bc
B73 grain		3.3 b	36.4 ab	4.2 b	25.3 c
mean		4.5 ± 0.9	35.6 ± 1.7	3.5 ± 0.5	27.5 ± 2.3

[a] Means in the same column followed by the same letter are not significantly different ($p < 0.05$).

Table 7. Effect of Protease Treatment on Ethanol Yield and Residual *r*-Protein Content in DDGS Produced from Dry-Milled Endosperm of Transgenic Grains[a]

feedstock/corn line	ethanol concn (g/L)	ethanol yield (%)	*r*-protein (μg/g)
r-CIα1 grain	152.4 b	33.9 b	nd[b]
r-GFP-Glb1 grain	160.7 a	35.7 a	nd
r-LTB grain	147.5 b	32.8 b	nd
r-GFP-Zn27 grain	163.7 a	36.4 a	nd
mean	155.6 ± 8.2	34.7 ± 1.6	nd

[a] Protease (Protex 6L, 0.05% on corn weight basis) was added during simultaneous saccharification and fermentation (SSF) stage of ethanol production. Means in the same column followed by the same letter are not significantly different ($p < 0.05$). [b] Not detected.

additional fatty acids for growth during fermentation (*21*). Third, no residual glucose was found in the finished beer, indicating undisturbed function of yeast throughout fermentation. Therefore, we attributed endosperm-rich fractions producing slightly lower conversion of starch to ethanol to (i) suboptimal conditions of starch liquefaction when using a feedstock containing higher than normal starch concentration or (ii) inhibitory effects on yeast caused by the higher than normal ethanol concentration produced when using starch-rich endosperm solids. Although yeast can tolerate 23% ethanol concentration, the ethanol production rate begins to decrease above 9% ethanol concentration (*21*).

The present study indicated that *r*-protein production could be integrated into existing dry-grind corn ethanol refineries. After extraction of the *r*-protein, the starch-rich spent solids could be used as fermentation feedstock to produce fuel ethanol and other industrial chemicals (*21*). The dry-milled germ contained 12−16% (db) oil in 19−25% of the corn mass, which was equivalent to about 70% recovery of the total corn oil. The germ oil of these transgenic corn lines may not be accepted for food use, but could be used to produce biodiesel or oleochemicals. Wet-milling produces quite pure germ (> 52% db fat) and low-protein starch (< 0.3% db) (*11*, *15*, *23*), but the process is energy-, water- and capital-intensive. Dry-milling (front-end degerming), on the other hand, produces fractions of lesser purities than wet-milling but may be more cost-effective.

The present study indicates that *r*-protein-lean coproducts can be utilized for fuel ethanol production after extraction of *r*-proteins. *r*-Proteins did not negatively affect ethanol production; similarly, the residual *r*-protein extraction buffer or germ removal did not negatively affect fermentation of the starch-rich solids. No functionally active *r*-GFP and *r*-LTB were found in DDGS; however, a small quantity of residual denatured *r*-CIα1 was detected in the DDGS of at least one event. Therefore, the safety of DDGS produced from transgenic grain for *r*-protein production needs to be evaluated in each case. Protease treatment of fermentation feedstock hydrolyzed the *r*-CIα1 and ensured that DDGS was free of any residual *r*-protein.

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